Serological Properties and Immunobiological Activities of Lipopolysaccharides from Black-pigmented and Related Oral *Bacteroides* Species

By TAKU FUJIWARA,¹ TATSUJI NISHIHARA,¹ TOSHIHIKO KOGA¹
AND SHIGEYUKI HAMADA^{2*}

¹Department of Dental Research, The National Institute of Health, Kamiosaki, Shinagawa-ku, Tokyo, 141, Japan

²Department of Oral Microbiology, Osaka University Faculty of Dentistry, Yamadaoka, Suita-Osaka, 565, Japan

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Lipopolysaccharides (LPS) from five species of oral *Bacteroides*, *B. gingivalis* strains 381 and ATCC 33277, *B. oralis* ATCC 33269, *B. loescheii* ATCC 15930, *B. intermedius* ATCC 25611 and *B. corporis* ATCC 33547, were extracted from whole cells by the phenol/water procedure, and subsequently purified by treatment with nuclease and ultracentrifugation. The LPS were composed of hexoses, glucosamine, fatty acids and phosphorus. Heptose and 2-keto-3-deoxyoctonate were not detected. The LPS preparations from *B. gingivalis* strains 381 and ATCC 33277 presented very similar SDS-polyacrylamide gel electrophoresis patterns when stained with ammoniacal silver. They produced a fused precipitin band against an antiserum to *B. gingivalis* 381 LPS in immunodiffusion tests. Antisera raised against the LPS from *B. loescheii* and *B. intermedius* reacted with the LPS prepared from all the oral *Bacteroides* strains except those of *B. gingivalis*. All the LPS preparations were mitogenic for spleen cells of BALB/c (nu/nu) mice, but not for thymus cells from C3H/HeN mice. The LPS induced marked mitogenic responses and polyclonal B cell activation for spleen cells of not only C3H/HeN (LPS responder) mice, but also C3H/HeJ (LPS nonresponder) mice. The mitogenic responses were not suppressed significantly upon addition of polymyxin B to the reaction mixture. These LPS also enhanced interleukin-1 production by murine peritoneal macrophages and mouse cell line J744.1 macrophages. Hydrolysis of *B. gingivalis* ATCC 33277 LPS in 1 M-HCl at 100 °C for 1 h yielded lipid and polysaccharide. The lipid portion was largely composed of fatty acids and glucosamine, and was mitogenic for spleen cells from C3H/HeJ as well as C3H/HeN mice, while the polysaccharide portion induced no significant mitogenic responses under similar experimental conditions.

INTRODUCTION

It has been suggested that dental plaque in the subgingival crevice is the major aetiological factor in the development of periodontal disease. Certain species of black-pigmented oral *Bacteroides* are suspected to be major causative agents of adult-type periodontitis, the most prevalent type of periodontal disease in the elderly. Among these, *Bacteroides gingivalis* and *B. intermedius* have been considered highly important in the pathogenesis of advancing periodontitis (Duerden *et al.*, 1987; Mayrand & Holt, 1988; Slots & Listgarten, 1988). The major habitat for these bacterial species in the oral cavity is the subgingival crevice area. Some cellular

Abbreviations: Con A, concanavalin A; FCS, foetal calf serum; HBSS, Hanks' balanced salt solution; IL-1, interleukin-1; KDO, 2-keto-3-deoxyoctonate (3-deoxy-D-manno-2-octulosonic acid); LPS, lipopolysaccharide; PFC, plaque-forming cells; SRBC, sheep red blood cells.

and extracellular components of oral *Bacteroides* species may occasionally induce inflammatory responses in the periodontal tissues, leading to the destruction of the tissues. A ubiquitous cellular component of Gram-negative bacteria, including oral *Bacteroides* species, is endotoxin lipopolysaccharide (LPS). LPS can elicit a plethora of immunobiological and pharmacological effects in host tissues, including the periodontal tissues (Daly *et al.*, 1980; Morrison, 1983; Westphal *et al.*, 1985). Higher levels of LPS have been demonstrated in extracts of dental plaque and root surface materials from patients with periodontal disease than from healthy controls (Daly *et al.*, 1980; Wilson *et al.*, 1986).

The chemical composition and some biological properties of the LPS from oral and non-oral *Bacteroides* species have been reported by several groups, demonstrating that both black-pigmented and non-black-pigmented *Bacteroides* species possess rather unusual LPS in terms of their chemical compositions and biological activities (Hofstad, 1974; Mansheim *et al.*, 1978; Koga *et al.*, 1985). However, no comparative examination of immunobiological properties of the various LPS from different species and strains of black-pigment forming *Bacteroides* of oral origin has been attempted. In this paper, we report results of immunochemical characterization and immunobiological studies on mitogenicity, polyclonal B cell activation, and interleukin (IL)-1 production of the LPS from several oral *Bacteroides* species in lymphoid cell cultures from LPS-responder and nonresponder mice.

METHODS

Bacterial strains and culture conditions. *B. gingivalis* ATCC 33277, *B. oralis* ATCC 33269, *B. loescheii* ATCC 15930, *B. intermedius* ATCC 25611 and *B. corporis* ATCC 33547 were from our culture collection. Organisms of each test strain were cultured anaerobically in a complex medium supplemented with haemin (5 mg l⁻¹) and menadione (1 mg l⁻¹) at 37 °C for 2–4 d, as described by Koga *et al.* (1985). After cultivation, cells were harvested by centrifugation, washed with pyrogen-free water, and lyophilized.

Preparation of LPS. LPS was extracted from lyophilized cells of the above strains by the phenol/water method (Westphal & Jann, 1965). The crude extract dissolved in pyrogen-free water was collected by ultracentrifugation at 100 000 g for 3 h. The resulting pellet was treated with nuclease P1 (Yamasa Shoyu Co.) and purified by repeated ultracentrifugations as described previously (Koga *et al.*, 1985). Chemical analyses were done as described by Koga *et al.* (1985). In some instances, the LPS preparation was retreated with phenol/water up to four times in order to obtain purer preparations. The lipid and the polysaccharide portions of LPS were obtained by hydrolysing LPS (1 mg ml⁻¹) in 1 M-HCl at 100 °C for 1 h, and chemical analyses were done as described above. The LPS of *B. gingivalis* 381 and *Escherichia coli* K235 were also used for reference in some experiments. The chemical characterization of these two LPS preparations was reported by Koga *et al.* (1985).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-polyacrylamide slab gels, with 12.5% (w/v) resolving and 4% (w/v) stacking gels, were prepared as described by Laemmli (1970). LPS (100 µg) was heated at 100 °C for 5 min in 10 mM-Tris/HCl buffer (pH 8.0) containing 2.5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 1 mM-EDTA, and 20% (v/v) glycerol. Electrophoresis was performed at 150 V constant voltage for 5 h. The LPS bands were visualized by staining with ammoniacal silver (Tsai & Frasch, 1982) or Coomassie brilliant blue.

Immunodiffusion. The Ouchterlony method of double diffusion in Noble agar (Difco) was performed. Antisera against LPS of *B. gingivalis* 381, *B. loescheii* and *B. intermedius* were obtained from New Zealand White rabbits which had been immunized by intramuscular injections of each LPS. LPS (1 mg) was suspended in 1 ml saline and mixed with an equal volume of Freund's complete adjuvant (Difco), and injected into the dorsal muscles of both sides of the trunk. This injection was repeated three times at 2-week intervals. After the immunization, the rabbits were bled from the central artery of the ear. The antisera were stored at -20 °C before use.

Mice. BALB/c (nu/nu), C3H/HeN and C3H/HeJ mice (all male) were obtained from the Department of Dental Research, The National Institute of Health, Tokyo. Spleen cells were obtained from mice aged 6–12 weeks; thymus cells were obtained from mice aged 4–6 weeks.

Lymphoid cell cultures. Mouse spleen or thymus cells were prepared by gently homogenizing spleens or thymuses in RPMI 1640 (Gibco) containing penicillin (100 U ml⁻¹), streptomycin (0.1 mg ml⁻¹), L-glutamic acid (2 mM) and HEPES buffer (15 mM, pH 7.2). This supplemented RPMI 1640 medium is referred to as RPMI-1640S. Cells (7 × 10⁵) were cultured in 0.2 ml RPMI-1640S with stimulants at 37 °C for 48 h in a 96-well plate in a humidified atmosphere of air/CO₂ (95:5, v/v). Cultures were pulsed for the final 6 h incubation with [³H]thymidine (0.25 µCi, 9.25 kBq; Amersham). The cultures were then harvested onto glass-fibre strips with a multiple cell harvester, and counted in an Aloka scintillation counter. All stimulants were tested in triplicate on three separate occasions.

To determine the effect of polymyxin B on mitogenic responses of mouse spleen cells, cells (7 × 10⁵) were cultured at 37 °C for 48 h in 0.2 ml RPMI-1640S containing 0–5 µg polymyxin B (Sigma) and 5 µg stimulant LPS. The incorporation of [³H]thymidine was quantified as described above.

Polyclonal B cell activation by LPS was assayed in spleen cells cultured *in vitro*, as follows. Spleen cells (1×10^6) were suspended in 0.2 ml RPMI-1640S supplemented with 2-mercaptoethanol (50 μ M) and foetal calf serum (FCS; 10%, v/v), and cultured with stimulant at 37 °C for 96 h in a 96-well plate in a humidified atmosphere of air/CO₂ (95:5, v/v). Cells were then washed with Hanks' balanced salt solution (HBSS; Gibco), and tested for direct anti-sheep-erythrocyte plaque-forming cell (anti-SRBC PFC) responses by the haemolytic plaque assay technique of Cunningham & Szenberg (1968).

IL-1 assay in macrophage cultures. Peritoneal macrophages were induced by intraperitoneal injection of 1.5 ml thioglycollate medium (Difco) into C3H/HeN mice. Peritoneal exudate cells were collected 3 d later by peritoneal lavage with 5 ml HBSS containing 1% (v/v) FCS (HBSS-FCS), washed, and resuspended to a density of 1×10^6 cells ml⁻¹ in RPMI-1640S containing 5% (v/v) FCS. The murine macrophage cell line J744.1 was kindly provided by Drs K. S. Akagawa and T. Tokunaga, The National Institute of Health, Tokyo. The cells were cultured in RPMI-1640S containing 10% (v/v) FCS at 37 °C in air/CO₂ (95:5, v/v). The adherent cells were removed with a rubber policeman, washed with HBSS and resuspended to a density of 1×10^6 cells ml⁻¹ in RPMI-1640S containing 5% (v/v) FCS.

The suspensions of peritoneal exudate cells or J744.1 cells (1 ml) were cultured in a 24-well plate (Corning) for 3 h at 37 °C, and nonadherent cells were washed off with HBSS-FCS. The adherent cells were incubated in RPMI-1640S with or without stimulant at 37 °C for 24 h in air/CO₂ (95:5, v/v). Supernatants were collected by centrifugation, filter-sterilized, and used for IL-1 assay. IL-1 activity in serial dilutions of the supernatant of the macrophage culture was determined by measuring the uptake of [³H]thymidine into C3H/HeJ mouse thymus cells (1.5×10^6) that were cultured in RPMI-1640S containing 10% (v/v) FCS and a submitogenic dose of Con A (1 μ g ml⁻¹; Sigma) in air/CO₂ (95:5, v/v). They were pulsed with 0.5 μ Ci (18.5 kBq) [³H]thymidine during the final 18 h of a 72 h incubation period.

RESULTS

SDS-PAGE of *Bacteroides* LPS

The silver-staining profile of the LPS preparations from the five oral *Bacteroides* species studied is shown in Fig. 1. Each showed a series of closely spaced bands in a ladder pattern. The LPS profiles of *B. gingivalis* strains 381 and ATCC 33277 were almost identical. There were some intense low-molecular-mass bands in the LPS of *B. oralis*, *B. intermedius*, and *B. corporis*. No protein bands were stained with Coomassie blue after SDS-PAGE of the purified LPS (100 μ g each) preparations (results not shown).

Serological properties of *Bacteroides* LPS

Immunodiffusion patterns of LPS preparations from the oral *Bacteroides* species are shown in Fig. 2. The LPS from *B. gingivalis* strains 381 and ATCC 33277 formed a fused precipitin band with antisera raised against LPS from *B. gingivalis* 381, but no precipitin band was developed with antisera to LPS from other species. On the other hand, the LPS from *B. oralis*, *B. loescheii*, *B. intermedius* and *B. corporis* formed one or two precipitin bands against antisera raised against the LPS from *B. loescheii* and *B. intermedius*, respectively. Further, an additional precipitin band was seen when the antiserum raised against LPS from *B. loescheii* was reacted with the homologous LPS.

Immunobiological activities of *Bacteroides* LPS

The LPS preparations from *B. gingivalis* (two strains), *B. oralis*, *B. loescheii*, *B. intermedius* and *B. corporis* were mitogenic for spleen cells of BALB/c (nu/nu) mice but not for thymus cells of C3H/HeN mice (Table 1). In the control experiments, *E. coli* K235 LPS and Con A induced selective mitogenic responses in BALB/c (nu/nu) spleen cells and C3H/HeN thymus cells, respectively. These results indicate that the LPS from oral *Bacteroides* species are B cell mitogens.

LPS from all five *Bacteroides* species were similarly mitogenic for spleen cells from both C3H/HeN and C3H/HeJ mice (Table 2). The maximal mitogenic responses were obtained with LPS doses of 5–10 μ g per well. The *E. coli* K235 LPS induced significant mitogenic responses in spleen cells of C3H/HeN mice, but not in those of C3H/HeJ mice. Polymyxin B, a cationic antibiotic, suppresses the mitogenic effect of enterobacterial LPS in murine spleen cell cultures. The mitogenicity of *E. coli* LPS was markedly inhibited upon addition of increasing

Fig. 1. Silver-stained gel after SDS-PAGE of LPS from oral *Bacteroides* species. Electrophoresis was performed in 12.5% (w/v) acrylamide gel, containing 100 µg LPS from *B. gingivalis* 381 (lane 1), *B. gingivalis* ATCC 33277 (lane 2), *B. oralis* ATCC 33269 (lane 3), *B. loescheii* ATCC 15930 (lane 4), *B. intermedius* ATCC 25611 (lane 5) and *B. corporis* ATCC 33547 (lane 6).

concentrations of polymyxin B. However, the *Bacteroides* LPS were only weakly affected by polymyxin B in terms of mitogenicity under similar experimental conditions (results not shown). These results, taken together, clearly suggest that the entity of *Bacteroides* LPS responsible for the mitogenic effect on murine spleen cells is different from that of enterobacterial LPS. The mitogenic activity of *B. gingivalis* ATCC 33277 LPS in C3H/HeJ spleen cell culture was retained even after repeated treatment of the LPS preparation with phenol/water.

In addition to mitogenic activity, the *Bacteroides* LPS induced *in vitro* polyclonal B cell activation responses, as revealed by an increase in the number of anti-SRBC PFC in spleen cell cultures from C3H/HeJ and C3H/HeN mice (Fig. 3). *E. coli* K235 LPS induced polyclonal B cell activation responses in C3H/HeN spleen cell cultures but not in C3H/HeJ spleen cell cultures.

The *Bacteroides* LPS stimulated both peritoneal macrophages of C3H/HeN mice and cells of mouse macrophage cell line J744.1, resulting in the induction of IL-1 in the supernatants of these cell cultures (Table 3). The culture supernatants, at up to eightfold dilution, clearly enhanced the uptake of [³H]thymidine into the thymus cells cultured in RPMI-1640S with 10% (v/v) FCS and a submitogenic dose (1 µg ml⁻¹) of Con A.

Isolation of the lipid portion from LPS

The LPS from *B. gingivalis* ATCC 33277 was treated in 1 M-HCl at 100 °C for 1 h to dissociate the lipid A and polysaccharide portions of the LPS. The lipid portion was mainly composed of fatty acids (70.5% w/w), and glucosamine (22.5%, w/w); no hexose was found. However, treatment of the LPS with 1% (v/v) or 5% (v/v) acetic acid at 100 °C for up to 5 h did not result in significant amounts of insoluble precipitate. The lipid portion of *B. gingivalis* ATCC 33277 LPS was mitogenic for spleen cells of both C3H/HeN and C3H/HeJ mice, while the lipid A prepared

Fig. 2. Immunodiffusion pattern of LPS from oral *Bacteroides* species with antisera raised against *B. gingivalis* 381 LPS (centre well A), *B. loeschii* ATCC 15930 LPS (centre well B), and *B. intermedius* ATCC 25611 (centre well C). The outer wells contained 7 μ l of LPS solutions (1 mg ml⁻¹) from the following strains: *B. gingivalis* 381 (1), *B. intermedius* ATCC 33269 (3), *B. loeschii* ATCC 15930 (4), *B. oralis* ATCC 33277 (2), *B. oralis* ATCC 25611 (5), and *B. corporis* ATCC 33547 (6).

Fig. 3. Polyclonal B cell activation by LPS from *Bacteroides* species in spleen cell cultures from C3H/HeN (a) and C3H/HeJ (b) mice. Spleen cells (1×10^6 per well) were incubated for 4 d with 0.02 μg (□), 0.2 μg (▨) or 2 μg (▩) LPS per well, or without LPS (■), in RPMI-1640S supplemented with 2-mercaptoethanol (50 μM) and 10% (v/v) FCS. After 4 d incubation, anti-SRBC PFC responses were determined as described in Methods. Values are expressed as the mean $\pm$ SD of triplicate cultures.

Table 1. Mitogenic responses of spleen cells from BALB/c (nu/nu) mice and thymus cells from C3H/HeN mice to LPS from oral *Bacteroides* species

Spleen cells (7×10^5 per well) from BALB/c (nu/nu) mice and thymus cells (7×10^5 per well) from C3H/HeN mice were incubated with LPS at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 in air. Cultures were pulsed with 0.25 μCi [^3H]thymidine during the last 6 h of a 48 h incubation period. Values are expressed as the mean $\pm$ SD of three different cultures.

Mitogen	Dose (μg per well)	[^3H]Thymidine uptake (c.p.m.)	
		BALB/c (nu/nu) spleen cells	C3H/HeN thymus cells
None	—	1020 $\pm$ 248	240 $\pm$ 24
Con A	5	1520 $\pm$ 209	24187 $\pm$ 1574
	1	1596 $\pm$ 68	1185 $\pm$ 72
<i>E. coli</i> K235 LPS	10	6492 $\pm$ 427	363 $\pm$ 8
	5	13151 $\pm$ 699	303 $\pm$ 67
	1	12793 $\pm$ 1107	387 $\pm$ 93
<i>B. gingivalis</i> 381 LPS	10	9560 $\pm$ 1889	245 $\pm$ 30
	5	17320 $\pm$ 1862	405 $\pm$ 51
	1	9229 $\pm$ 849	312 $\pm$ 82
<i>B. gingivalis</i> ATCC 33277 LPS	10	7882 $\pm$ 1007	378 $\pm$ 3
	5	5440 $\pm$ 204	169 $\pm$ 55
	1	4775 $\pm$ 491	182 $\pm$ 30
<i>B. oralis</i> ATCC 33269 LPS	10	16100 $\pm$ 933	180 $\pm$ 23
	5	14602 $\pm$ 892	188 $\pm$ 15
	1	8799 $\pm$ 304	150 $\pm$ 11
<i>B. loescheii</i> ATCC 15930 LPS	10	12793 $\pm$ 2642	201 $\pm$ 44
	5	16062 $\pm$ 1578	300 $\pm$ 53
	1	8799 $\pm$ 304	150 $\pm$ 11
<i>B. intermedius</i> ATCC 25611 LPS	10	31348 $\pm$ 2748	141 $\pm$ 10
	5	28952 $\pm$ 1908	120 $\pm$ 19
	1	6021 $\pm$ 592	150 $\pm$ 25
<i>B. corporis</i> ATCC 33547 LPS	10	10124 $\pm$ 642	142 $\pm$ 27
	5	7495 $\pm$ 1492	174 $\pm$ 105
	1	3097 $\pm$ 687	76 $\pm$ 17

Table 2. Mitogenic responses of spleen cells from C3H/HeN and C3H/HeJ mice to LPS from oral *Bacteroides* species

Spleen cells (7×10^5 per well) from C3H/HeN and C3H/HeJ mice were incubated with LPS at 37 °C in a humidified atmosphere of 5% (v/v) CO₂ in air. Cultures were pulsed with 0.25 µCi [³H]thymidine during the last 6 h of a 48 h incubation period. Values are expressed as the mean $\pm$ SD of three different cultures.

Mitogen	Dose (µg per well)	[³ H]Thymidine uptake (c.p.m.) by spleen cells from:	
		C3H/HeN	C3H/HeJ
None	–	397 $\pm$ 138	413 $\pm$ 39
<i>E. coli</i> K235 LPS	10	20769 $\pm$ 1025	729 $\pm$ 57
	5	25138 $\pm$ 3561	976 $\pm$ 78
	1	24956 $\pm$ 2321	873 $\pm$ 61
<i>B. gingivalis</i> 381 LPS	10	12809 $\pm$ 692	16212 $\pm$ 772
	5	16461 $\pm$ 1190	24840 $\pm$ 2208
	1	13098 $\pm$ 246	17809 $\pm$ 1243
<i>B. gingivalis</i> ATCC 33277 LPS	10	16461 $\pm$ 1191	18322 $\pm$ 917
	5	13518 $\pm$ 1165	15573 $\pm$ 983
	1	6267 $\pm$ 706	7522 $\pm$ 448
<i>B. oralis</i> ATCC 33269 LPS	10	17430 $\pm$ 1607	10630 $\pm$ 578
	5	22039 $\pm$ 3235	11573 $\pm$ 528
	1	16065 $\pm$ 673	10932 $\pm$ 500
<i>B. loescheii</i> ATCC 15930 LPS	10	20760 $\pm$ 358	19862 $\pm$ 2623
	5	20097 $\pm$ 1115	25291 $\pm$ 2101
	1	14889 $\pm$ 1717	20004 $\pm$ 1268
<i>B. intermedius</i> ATCC 25611 LPS	10	48030 $\pm$ 4426	14820 $\pm$ 1498
	5	32870 $\pm$ 7144	13956 $\pm$ 1335
	1	12541 $\pm$ 358	8204 $\pm$ 204
<i>B. corporis</i> ATCC 33547 LPS	10	16014 $\pm$ 1126	17992 $\pm$ 956
	5	10330 $\pm$ 63	14307 $\pm$ 1454
	1	5007 $\pm$ 830	6614 $\pm$ 363

Table 3. Effect of LPS from oral *Bacteroides* species on IL-1 production by C3H/HeN peritoneal macrophages or cells of mouse macrophage cell line J744.1

Peritoneal macrophages (1×10^6 per well) from C3H/HeN mice, or cells of mouse macrophage cell line J744.1 (1×10^6 per well), were incubated for 1 d with LPS (25 µg) in 1 ml RPMI 1640 medium. The macrophage culture supernatant and thymus cells (1.5×10^6) from C3H/HeJ mice were then cultured in 200 µl RPMI 1640 supplemented with a submitogenic dose of Con A ($1 \mu\text{g ml}^{-1}$). Cultures were pulsed with 0.5 µCi [³H]thymidine during the last 18 h of a 72 h incubation period. Values are expressed as the mean $\pm$ SD of three different cultures.

LPS from	[³ H]Thymidine uptake (c.p.m.) by thymus cells					
	Dilution of culture supernatant of C3H/HeN peritoneal macrophages			Dilution of culture supernatant of macrophage cell line J744.1 cells		
	1/2	1/4	1/8	1/2	1/4	1/8
None	218 $\pm$ 8	289 $\pm$ 97	238 $\pm$ 27	421 $\pm$ 28	354 $\pm$ 50	306 $\pm$ 25
<i>B. gingivalis</i> 381	3412 $\pm$ 373	1522 $\pm$ 216	751 $\pm$ 98	4221 $\pm$ 522	2583 $\pm$ 28	938 $\pm$ 189
<i>B. gingivalis</i> ATCC 33277	2847 $\pm$ 281	1448 $\pm$ 116	869 $\pm$ 74	4820 $\pm$ 238	2233 $\pm$ 319	1498 $\pm$ 168
<i>B. oralis</i> ATCC 33269	3612 $\pm$ 414	2964 $\pm$ 510	1126 $\pm$ 333	3605 $\pm$ 32	2132 $\pm$ 204	1169 $\pm$ 196
<i>B. loescheii</i> ATCC 15930	3248 $\pm$ 499	1624 $\pm$ 264	864 $\pm$ 27	4456 $\pm$ 14	3076 $\pm$ 67	1278 $\pm$ 298
<i>B. intermedius</i> ATCC 25611	3429 $\pm$ 801	2075 $\pm$ 75	1660 $\pm$ 710	4554 $\pm$ 270	2583 $\pm$ 42	1540 $\pm$ 220
<i>B. corporis</i> ATCC 33547	3477 $\pm$ 699	2221 $\pm$ 438	719 $\pm$ 116	3770 $\pm$ 214	2692 $\pm$ 18	2653 $\pm$ 242

from *E. coli* K235 LPS was mitogenic only for C3H/HeN spleen cells, not for C3H/HeJ spleen cells. The polysaccharide portion of the LPS from *B. gingivalis* ATCC 33277 and *E. coli* K235 did not exhibit significant mitogenic activity for the murine spleen cells.

DISCUSSION

LPS prepared by phenol/water extraction of whole cells of *B. gingivalis* was significantly different from that of *Enterobacteriaceae* species such as *E. coli* in terms of chemical composition, immunobiological activities, and toxicities (Koga *et al.*, 1985, 1987). LPS of *B. gingivalis*, *B. intermedius* and *B. fragilis* are basically similar and possess no significant quantities of heptose and KDO (Hofstad, 1974; John & Bryn, 1986; Koga *et al.*, 1985; Mansheim *et al.*, 1978). All the LPS preparations from the *Bacteroides* species used in this study possess various quantities of hexose, glucosamine, fatty acid and phosphorus, but no detectable heptose or KDO (results not shown). The presence of 'O-phosphorylated KDO' in *B. gingivalis* LPS has recently been reported by Kumada *et al.* (1988).

The profile of LPS from *Bacteroides* species in SDS-PAGE stained with ammoniacal silver gave regularly spaced ladder patterns with sugar chain lengths of varying molecular size (Fig. 1). A rather different result has been reported for *B. gingivalis* LPS by Naito *et al.* (1985), who found broad and heavily staining bands of lower molecular sizes. The presence of closely spaced bands on the silver-stained SDS-PAGE is suggestive of the pattern produced by the smooth type of LPS from *Enterobacteriaceae* species (Weintraub *et al.*, 1985).

Nair *et al.* (1983) described LPS from *B. gingivalis* producing a precipitin band against antisera to strains of this species in immunodiffusion. Similar results were obtained in this study using the antiserum to *B. gingivalis* LPS (Fig. 2). The LPS from other *Bacteroides* species did not yield a precipitin band under the same experimental condition. Millar *et al.* (1987) have recently reported that a monoclonal antibody to the LPS of *B. gingivalis* 381 was reactive with LPS from this species, but not with that from other *Bacteroides* species. Furthermore, antibodies to LPS from *B. loescheii* and *B. intermedius* produced precipitin bands against LPS from these *Bacteroides* species as well as *B. oralis* and *B. corporis*. These findings clearly show the presence of some common antigen among these species (Fig. 2). The chemical linkage of the polysaccharide portion of these LPS responsible for immunochemical specificity remains to be elucidated. These results seem to indicate that *B. gingivalis* LPS possess a species-specific immunochemical specificity.

Variable results have been reported from different laboratories concerning the immunological and biological activities of LPS of oral and non-oral *Bacteroides* species using LPS-responsive and nonresponsive murine strains, C3H/HeN and C3H/HeJ, respectively. Joiner *et al.* (1982) first suggested that the phenol/water-extracted LPS from *B. fragilis* ATCC 23475 stimulated lymphoid cells of both C3H/HeN and C3H/HeJ mice. Our previous and present results basically coincide with those of Joiner *et al.* (1982). Nair *et al.* (1983), on the other hand, reported that *B. gingivalis* 381 LPS was not mitogenic for spleen cells of either mouse strain, although the LPS induced Ca^{2+} release from organ culture of bones. In their experiments, they included 10% (v/v) FCS in the mitogenesis assay system. In our experimental conditions, the incorporation of as much as 10% (v/v) FCS suppressed the mitogenic activity of *B. gingivalis* LPS as compared to that in the culture medium without FCS (results not shown). This finding may suggest that at least some batches of FCS contain activity that inhibits the mitogenic potential of *B. gingivalis* LPS. Ulevitch *et al.* (1979) reported that a serum lipoprotein could bind to LPS, resulting in a decreased endotoxicity of the LPS. Natural antibodies reactive with the test sample of LPS may also participate in this phenomenon.

The possibility that some contaminant may be present in the phenol/water-extracted LPS preparations from *Bacteroides* species must be considered. In this connection, we found that phenol/water extracts of *Fusobacterium nucleatum* exhibited a significant mitogenicity in C3H/HeJ spleen cell cultures (Okahashi *et al.*, 1988). However, this material contained a relatively high amount of protein (>5%, w/v). Repeated extractions of the LPS preparation with hot phenol/water yielded a more refined protein-free LPS preparation that showed

mitogenic activity in C3H/HeN but not C3H/HeJ spleen cell cultures (Okahashi *et al.*, 1988). However, even after four repeated extractions with phenol/water, the *Bacteroides* LPS still induced significant mitogenic responses in C3H/HeJ as well as C3H/HeN spleen cell cultures. These results suggest that the mitogenic potential of the *Bacteroides* LPS in C3H/HeJ spleen cell cultures is an intrinsic property of their endotoxic molecule. Another unique property of LPS from *Bacteroides* species is that polymyxin B does not significantly suppress their mitogenic activities. The structural entity of LPS from *Bacteroides* species responsible for these properties is not known; the absence of KDO and heptose but possible presence of *O*-phosphorylated KDO in *Bacteroides* LPS may contribute to differences in biological responses between them and enterobacterial LPS.

The active entity of enterobacterial LPS is endotoxic lipid A, as confirmed recently by extensive studies using synthetic analogues of lipid A (Westphal *et al.*, 1985). The lipid portion of the LPS from bacteria of the *Enterobacteriaceae* can be isolated from native LPS by mild hydrolysis in 1% (v/v) acetic acid at 100 °C for 2–4 h (Galanos *et al.*, 1971). Preliminary experiments indicated that hydrolysis in HCl, rather than acetic acid, was the most appropriate method to isolate the lipid portion from *Bacteroides* LPS. The lipid portion thus obtained exhibited mitogenic responses in both C3H/HeN and C3H/HeJ spleen cells, although the level of stimulation by the lipid portion was not as high as that induced by the whole LPS.

REFERENCES

- CUNNINGHAM, A. J. & SZENBERG, A. (1968). Further improvements in the plaque technique for detecting single antibody-forming cells. *Immunology* **14**, 599–600.
- DALY, C. G., SEYMOUR, G. J. & KIESER, J. B. (1980). Bacterial endotoxin: a role in chronic inflammatory periodontal disease? *Journal of Oral Pathology* **9**, 1–15.
- DUERDEN, B. I., GOODWIN, L. & O'NEIL, T. C. A. (1987). Identification of *Bacteroides* species from adult periodontal disease. *Journal of Medical Microbiology* **24**, 133–137.
- GALANOS, C., LÜDERITZ, O. & WESTPHAL, O. (1971). Preparation and properties of antisera against the lipid-A component of bacterial lipopolysaccharides. *European Journal of Biochemistry* **24**, 116–122.
- HOFSTAD, T. (1974). The distribution of heptose and 2-keto-3-deoxy-octonate in *Bacteroidaceae*. *Journal of General Microbiology* **85**, 314–320.
- JOHNE, B. & BRYN, K. (1986). Chemical composition and biological properties of a lipopolysaccharide from *Bacteroides intermedius*. *Acta pathologica, microbiologica et immunologica scandinavica* **B94**, 265–271.
- JOINER, K. A., MCADAM, K. P. W. J. & KASPER, D. L. (1982). Lipopolysaccharides from *Bacteroides fragilis* are mitogenic for spleen cells from endotoxin responder and nonresponder mice. *Infection and Immunity* **36**, 1139–1145.
- KOGA, T., NISHIHARA, T., FUJIWARA, T., NISIZAWA, T., OKAHASHI, N., NOGUCHI, T. & HAMADA, S. (1985). Biochemical and immunobiological properties of lipopolysaccharide (LPS) from *Bacteroides gingivalis* and comparison with LPS from *Escherichia coli*. *Infection and Immunity* **47**, 638–647.
- KOGA, T., ODAKA, C., MORO, I., FUJIWARA, T., NISHIHARA, T., OKAHASHI, N. & HAMADA, S. (1987). Local Schwartzman activity of lipopolysaccharides from several selected strains of suspected periodontopathic bacteria. *Journal of Periodontal Research* **22**, 103–107.
- KUMADA, H., WATANABE, K., UMEMOTO, T., HAISHIMA, Y., KONDO, S. & HISATSUNE, K. (1988). Occurrence of *O*-phosphorylated 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Bacteroides gingivalis*. *FEMS Microbiology Letters* **51**, 77–80.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- MANSHEIM, B. J., ONDERDONK, A. B. & KASPER, D. L. (1978). Immunochemical and biologic studies of the lipopolysaccharide of *Bacteroides melaninogenicus* subspecies *asaccharolyticus*. *Journal of Immunology* **120**, 72–78.
- MAYRAND, D. & HOLT, S. C. (1988). Biology of asaccharolytic black-pigmented *Bacteroides* species. *Microbiological Reviews* **52**, 134–152.
- MILLAR, S. J., CHEN, P. B. & HAUSMANN, E. (1987). Monoclonal antibody for identification of *Bacteroides gingivalis* lipopolysaccharide. *Journal of Clinical Microbiology* **25**, 2437–2439.
- MORRISON, D. C. (1983). Bacterial endotoxins and pathogenesis. *Reviews of Infectious Diseases* **5**, S733–S747.
- NAIR, B. C., MAYBERRY, W. R., DZIAK, R., CHEN, P. B., LEVINE, M. J. & HAUSMANN, E. (1983). Biological effects of a purified lipopolysaccharide from *Bacteroides gingivalis*. *Journal of Periodontal Research* **18**, 40–49.
- NAITO, Y., OKUDA, K., KATO, T. & TAKAZOE, I. (1985). Monoclonal antibodies against surface antigens of *Bacteroides gingivalis*. *Infection and Immunity* **50**, 231–235.
- OKAHASHI, N., KOGA, T., NISHIHARA, T., FUJIWARA, T. & HAMADA, S. (1988). Immunological properties of lipopolysaccharides isolated from *Fusobacterium nucleatum* and *F. necrophorum*. *Journal of General Microbiology* **134**, 1707–1715.
- SLOTS, J. & LISTGARTEN, M. A. (1988). *Bacteroides gingivalis*, *Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in human periodontal diseases. *Journal of Clinical Periodontology* **15**, 85–93.
- TSAI, C. M. & FRASCH, C. E. (1982). A sensitive silver

- stain for detecting lipopolysaccharides in polyacrylamide gels. *Analytical Biochemistry* **119**, 115–119.
- ULEVITCH, R. J., JOHNSTON, A. R. & WEINSTEIN, D. B. (1979). New function for high density lipoproteins. *Journal of Clinical Investigation* **64**, 1516–1524.
- WEINTRAUB, A., ZÄHRINGER, U. & LINDBERG, A. A. (1985). Structural studies of the polysaccharide part of the cell wall lipopolysaccharide from *Bacteroides fragilis* NCTC 9343. *European Journal of Biochemistry* **151**, 657–661.
- WESTPHAL, O. & JANN, K. (1965). Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. *Methods in Carbohydrate Chemistry* **5**, 83–91.
- WESTPHAL, O., LÜDERITZ, O., GALANOS, C., MAYER, H. & RIETSCHEL, E. T. (1985). The story of bacterial endotoxin. In *Advances in Immunopharmacology*, pp. 13–34. Edited by L. Chedid, J. W. Hadden, F. Spreafico, P. Dukor & D. Willoughby. Oxford: Pergamon Press.
- WILSON, M., MOORE, J. & KIESER, J. B. (1986). Identity of limulus amoebocyte lysate-active root surface materials from periodontally involved teeth. *Journal of Clinical Periodontology* **13**, 743–747.